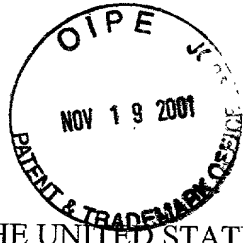


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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF

RYUICHIRO KURANE, et al.

:

GROUP:

SERIAL NO: 09/891,517

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EXAMINER:

FILED: JUNE 27, 2001

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FOR: NOVEL NUCLEIC ACID PROBES,
METHOD FOR DETERMINING
CONCENTRATIONS OF NUCLEIC
ACID BY USING THE PROBES,
AND METHOD FOR ANALYZING
DATA OBTAINED BY THE METHOD

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Responsive to the Official Correspondence dated July 17, 2001, Applicants submit herewith a substitute Abstract of the Disclosure, a substitute specification, a substitute Sequence Listing, and a corresponding computer-readable Sequence Listing. Prior to examination on the merits, please amend the above-identified application as follows.

IN THE ABSTRACT OF THE DISCLOSURE

Please replace the original abstract at page 197 with the substitute abstract attached hereto.

09/891,517

IN THE SPECIFICATION

Page 197 (Abstract), after the last line, beginning on the next page, please delete the original Sequence Listing attached at pages 1 to 23 and replace it with the substitute Sequence Listing attached hereto.

Please replace the original specification at pages 1 to 180 with the substitute specification attached hereto.

Please amend the substitute specification as follows:

Please replace the paragraph at page 7, lines 5-9, as follows:

--Performance of a polymorphous analysis on a target gene LJ after amplifying the target gene by a quantitative gene amplification method makes it possible to easily and quickly determine the pre-amplification amount and polymorphous composition of the target gene with good quantitateness.--

Please replace the paragraph at page 8, lines 3-16, as follows:

--2) A nucleic acid probe for determining a concentration of a target nucleic acid, the probe being labeled with a fluorescent dye, wherein:

the probe is labeled at an end portion thereof with the fluorescent dye, and

the probe has a base sequence designed such that, when the probe hybridizes at the end portion thereof to the target nucleic acid, at least one G (guanine) base exists in a base sequence of the target nucleic acid at a position 1 to 3 bases from an end base of the target nucleic acid hybridized with the probe;

whereby the fluorescent dye is reduced in fluorescence emission when the probe labeled with the fluorescent dye hybridizes to the target nucleic acid.--

Please replace the paragraph at page 14, lines 11-24, as follows:

--23) A method for determining an initial concentration of a target nucleic acid amplified in PCR, which comprises:

conducting reactions in PCR by using a nucleic acid probe as described above under any one of 1) to 6);

measuring an intensity of fluorescence in a reaction system in which in a course of a nucleic acid extending reaction, the probe has been degraded out from the probe-target nucleic acid complex by polymerase or in which a nucleic acid denaturing reaction is proceeding or has been completed and also an intensity of fluorescence in the reaction system in which the target nucleic acid or amplified target nucleic acid is hybridized with the nucleic acid probe; and then

calculating percentage of a change in the latter intensity of fluorescence from the former intensity of fluorescence.

Please replace the paragraph at page 52, lines 6-9, as follows:

--The nucleic acid probe according to the present invention can also be readily obtained by ordering its synthesis like the synthesis of the oligonucleotide, provided that only the designing of the probe can be completed.--

Please replace the paragraph at page 52, lines 10-12, as follows:

--A description will next be made about the fluorescence quenching probe according to the second aspect of the present.

This probe is characterized in that it is an oligonucleotide labeled with a single fluorescent dye and, when hybridized with a target nucleic acid, the intensity of its fluorescence decreases. It, therefore, has a property opposite to the fluorescence emitting probe.--

Please replace the paragraph at page 53, line 21, to page 54, line 15, as follows:

--The number of bases in the probe according to the present invention is similar to that in the above-described invention. No particular limitation is imposed on the base sequence of the probe insofar as it specifically hybridizes to the target nucleic acid. Preferred examples of the base sequence of the probe can include:

(1) a base sequence designed such that at least one G (guanine) base exists in the base sequence of the target nucleic acid at a position 1 to 3 bases from the end base portion of the target nucleic acid hybridized to the-probe,

(2) a base sequence designed such that plural base pairs of a nucleic acid hybrid complex forms at least one G (guanine) and C (cytosine) pair at an end portion of the probe, and

(3) a base sequence designed such that in the probe modified with the fluorescent label at a portion other than the 5' end phosphate group or the 3' end OH group, base pairs in the fluorescence-labeled portion forms at least one G (guanine) and C (cytosine) pair,

when the nucleic acid probe labeled with the fluorescent dye is hybridized with the target nucleic acid.--

Please amend the paragraph at page 61, line 12, to page 62, line 18, as follows:

--Fundamental operations in the determination method making use of the device according to the present invention are simply to place a solution, which contains a target nucleic acid such as mRNA, cDNA or rRNA, on the solid support on which the nucleic probes are bound and then to induce hybridization. As a result, a change in the intensity of fluorescence takes place corresponding to the concentration of the target nucleic acid, and the target nucleic acid can then be detected and quantitated from the change in the intensity of fluorescence. Further, binding of many nucleic acid probes of different base sequences on a surface of a single support makes it possible to determine concentrations of many target

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nucleic acids at the same time. As this device can be used for exactly the same application as a DNA chip, that is, for the determination of the concentrations of the target nucleic acids, it is a novel DNA chip. Under reaction conditions optimal for the target nucleic acid, the nucleic acids other than the target nucleic acid do not change the intensities of fluorescence emitted from the probe. No operation is, therefore, needed for washing off the unreacted nucleic acids. Further, independent temperature control of the individual nucleic acid probes according to the present invention by their corresponding microheaters makes it possible to control the probes under their optimal reaction conditions, respectively. Accurate determination of concentrations is therefore feasible. In addition, a denaturation curve between each nucleic acid probe of this invention and its corresponding target nucleic acid can be analyzed by continuously changing the temperature with the microheater and measuring the intensity of fluorescence during the changing of the temperature. From differences in such denaturation curves, it is possible to determine properties of the hybridized nucleic acid and also to detect SNP.--

Please replace the paragraph at page 66, lines 4-11, as follows:

--In the present invention, the nucleic acid probe is hybridized to the target nucleic acid as described above. The intensity of fluorescence emitted from the fluorescent dye is measured both before and after the hybridization, and a change in fluorescence intensity after the hybridization is then calculated. As the change is proportional to the concentration of the target nucleic acid, the concentration of the target nucleic acid can be determined.--

Please replace the paragraph at page 74, line 22, to page 75, line 7, as follows:

--The followings can be mentioned as illustrative examples of the fluorescence quenching probe:

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T06TTT 2TS1660

(1) A probe labeled, at an portion, preferably, an end thereof, with a fluorescent dye useful in the practice of the present invention. The base sequence of the probe is designed such that, when hybridizes to a target nucleic acid, at least one G (guanine) base exists in the base sequence of the target nucleic acid at a position 1 to 3 bases from the end base of the target nucleic acid hybridized on the end portion or end of the probe where the probe is labeled with the fluorescent dye.--

Please replace the paragraph at page 77, line 11, to page 78, line 22, as follows:

--In particular, the above-described probe (7) of the present invention is designed such that it is not used as a primer. PCR is conducted by using a single probe of the present invention as opposed to two (fluorescent-dye-labeled) probes needed in a real-time quantitative PCR method making use of the FRET phenomenon. The probe is added to a PCR reaction system, and PCR is then conducted. During a nucleic acid extending reaction, the probe which has been in a form hybridized with the target nucleic acid or amplified target nucleic acid is degraded by polymerase and is dissociated off from the nucleic acid hybrid complex. The intensity of fluorescence of the reaction system at this time or the reaction system in which a nucleic acid denaturing reaction has completed is measured. Further, the intensity of fluorescence of the reaction system in which the target nucleic acid or amplified target nucleic acid has hybridized with the probe (i.e., the reaction system at the time of an annealing reaction or at the time of the nucleic acid extending reaction until the probe is eliminated from the nucleic acid hybrid complex by polymerase) is measured. By calculating a decrease in the former fluorescence intensity based on the latter fluorescence intensity, the concentration of the amplified nucleic acid is determined. The intensity of fluorescence is high when the probe has completely dissociated from the target nucleic acid or amplified

target nucleic acid by the nucleic acid denaturing reaction or when the probe has been degraded out from the hybrid complex of the probe and the target nucleic acid or amplified nucleic acid at the time of extension of the nucleic acid. However, the intensity of fluorescence of the reaction system in which an annealing reaction has been completed and the probe has fully hybridized to the target nucleic acid or amplified target nucleic acid or of the reaction system until the probe is degraded out of the hybrid complex of the probe and the target nucleic acid or, amplified target nucleic acid by polymerase at the time of a nucleic acid extending reaction is lower than the former. The decrease in the intensity of fluorescence is proportional to the concentration of the amplified nucleic acid.--

Please replace the paragraph at page 86, line 22, to page 87, line 20, as follows:

--A third feature resides in a data analysis method, which comprises the following processing steps:

1) performing processing in accordance with the following formula (5), (6) or (7) by using data of rates or percentages of changes in fluorescence as calculated in accordance with said formula (3) or (4):

$$\log_b(F_n), \ln(F_n) \quad (5)$$

$$\log_b\{(1-F_n) \times A\}, \ln\{(1-F_n) \times A\} \quad (6)$$

$$\log_b\{(F_n-1) \times A\}, \ln\{(F_n-1) \times A\} \quad (7)$$

where

A, b: desired numerical values, preferably integers, more preferably natural numbers and, when A=100, b=10, $\{(F_n-1) \times A\}$ is expressed in terms of percentage (%), and

F_n : rate or percentage of a change in fluorescence in an n^{th} cycle as calculated in accordance with the formula (3) or formula (4),

2) determining a cycle in which said processed value of said processing step 1) has reached a constant value,

3) calculating a relational expression between cycle of a nucleic acid sample of a known concentration and the number of copies of said target nucleic acid at the time of initiation of a reaction, and

4) determining the number of copies of said target nucleic acid in an unknown sample upon initiation of PCR.--

Please replace the paragraph at page 92, lines 15-24, as follows:

--The feature of the polymorphous analysis method according to the present invention resides in the use of the nucleic acid probe of this invention in a conventional polymorphous analysis method to determine a nucleic acid. The term "polymorphous" or "polymorphism" as used herein means biological polymorphous or polymorphism. In the present invention, it means especially the polymorphism of a gene (RNA, DNA, gene) on which the polymorphism is brought about. It has the same meaning as commonly employed these days in molecular biology.--

Please replace the paragraph at page 94, line 25, to page 95, line 9, as follows:

--For example, as has been described in detail in connection with the second aspect of the invention, a fluorescence quenching probe is labeled at an end thereof with a fluorescent dye, and its base sequence is designed such that, when the probe hybridizes at the end portion thereof to a target gene, at least one G (guanine) base exists in a base sequence of the target gene at a position 1 to 3 bases from the portion of an end base pair of the target gene hybridized with the probe, whereby the fluorescent dye is reduced in fluorescence emission when the probe hybridizes to the target gene.--

Please replace the paragraph at page 99, lines 7-12, as follows:

--It is preferred to prepare a working line for the target gene by using an authentic target gene before the amplifying reaction of the target gene. A description will now be made about an illustrative case in which the above-described fluorescence quenching probe was used as a primer and the real-time monitoring quantitative PCR method was conducted.--

Please replace the paragraph at page 101, line 22, to page 102, line 1, as follows:

--(2) Gene fragments digested as described above can preferably be thermally denatured into single-stranded forms. This denaturation treatment can be conducted under usual conditions known to the public. For example, they are treated at 97°C for 5 minutes and then chilled in ice.--

Please replace the paragraph at page 107, line 20, to page 108, line 3, as follows:

--The invention nucleic acid probe synthesized as described above is free of any base sequence having complementation at at least two positions between the base chains at positions where the probe was labeled with Texas Red as a fluorescent dye and Dabcyl as a quencher substance, respectively. The invention nucleic acid probe, therefore, does not form any double-stranded chain in its own chain. In other words, the invention nucleic acid probe does not form any stem-loop structure.--

Please replace the paragraph at page 111, line 25, to page 113, line 1, as follows:

--The results are diagrammatically shown in FIG. 4. As is appreciated readily from the diagram, it was observed that in most of the fluorescence emitting probes of the present invention dually modified by Dabcyl and Texas Red, hybridization with the target deoxyribooligonucleotide leads to an increase in the emission of fluorescence compared with the emission of fluorescence before the hybridization. Further, maximum emission of fluorescence was observed when the inter-base distance from the base having the phosphate group labeled with Texas Red to the Dabcyl-labeled base (when counted by assuming that

the base number of the base labeled with Text Red was the 0th base) was 6 bases long. The emission of fluorescence at that time was about 11 times higher compared with that before hybridization. When the inter-base distance was 16 bases long, large emission of fluorescence was also observed. The emission of fluorescence at that time was about 11 times as in the case of the 6 bases. As a DNA helix makes a turn with 10 bases, the 6th and 16th bases of a double-stranded DNA structure as observed from the 5' end base are located substantially on the opposite side of the helix. It is therefore considered that, when the 6th and 16th bases were labeled with the quencher substance, quenching of fluorescence took place based on transfer of electrons between Dabcyl and Texas Red when the deoxyribooligonucleotide is in the single-stranded form, but as a result of physical separation of Dabcyl and Texas Red from each other by the hybridization, the quenching of fluorescence based on the transfer of electrons was canceled and Texas Red emitted fluorescence.--

Please replace the paragraph at page 113, line 13, to page 114, line 2, as follows:

--An absorption of Dabcyl as a quencher appears at 400 to 500 nm. Many of probes with large emission of fluorescence, however, emitted fluorescence at wavelengths substantially shifted from the absorption of Dabcyl, that is, at wavelengths longer than 550 nm. In the case of a fluorescent dye which emits fluorescence at wavelengths longer than 550 nm, the mechanism of fluorescence quenching by Dabcyl is considered to be attributable primarily to transfer of photoexcited electrons rather than FRET. Since Dabcyl and the fluorescent dye are physically separated from each other as a result of a change in the stereostructure of the double-stranded nucleic acids by hybridization, the fluorescence quenching phenomenon by the transfer of photoexcited electrons is cancelled. In the case of a fluorescent dye such as FITC which emits fluorescence at wavelengths close to the

absorption of Dabcyl, it is considered that, even when Dabcyl and a fluorescent dye are physically separated as a result of a change in the stereostructure of the double-stranded nucleic acid and the fluorescence quenching phenomenon based on transfer of photoexcited electrons is hence cancelled, no substantial emission of fluorescence takes place from quenching of fluorescence by FRET because the quenching of fluorescence by FRET prevails. Accordingly, a dye capable of satisfying the following three conditions is desired as a fluorescent dye for use in a fluorescence emitting probe according to the present invention:

(1) the fluorescence quenching phenomenon based on transfer of photoexcited electrons occurs between the fluorescent dye and Dabcyl; (2) Fluorescence of wavelengths substantially shifted from the absorption of Dabcyl is emitted; and (3) a strong interaction exists between the fluorescent dye and Dabcyl to reduce the intensity of fluorescence before hybridization (in other words, to further facilitate the occurrence of the fluorescence quenching phenomenon by transfer of photoexcited electrons).--

Please replace the paragraph at page 133, line 20, to page 134, line 2, as follows:

--Using a DNA synthesizer, an oligoribonucleotide having a base sequence of (5')TCCTTTGAGT TCCCGGCCGG(3') A was synthesized as in the above to provide it as a forward-type helper probe. On the other hand, an oligoriboxynucleotide having a base sequence of (5')CCCTGGTCGT AAGGGCCATG ATGACTTGAC GT (3') was synthesized by using a DNA synthesizer, in a similar manner as described above to provide it as a backward-type, in other words, reverse-type helper probe.--

Please replace the paragraph at page 145, line 15, to page 146, line 15, as follows:

--Four oligonucleotides with the below-described base sequences were synthesized using the same DNA synthesizer as that employed in Example 12. Further, an invention nucleic acid probe having the below-described base sequence was also synthesized in a

similar manner as in Example 12. The target oligonucleotides were separately hybridized with the probe in solutions. An investigation was then made as to whether or not a single base substitution can be determined from a change in fluorescence intensity. The base sequence of the nucleic acid probe according to the present invention is designed such that, if G exists at the 3' end of any one of the target oligonucleotides, it matches 100% with the base sequence of the particular oligonucleotide. The hybridization temperature was set at 40°C at which all base pairs between the probe and the target oligonucleotide can hybridize 100%. The concentrations of the probe and target oligonucleotides, the concentration of a buffer solution, a fluorimeter, fluorescence measuring conditions, experimental procedures, and the like were set or chosen as in Example 12.--

Please replace Table 7 on page 147 as follows:

--Table 7

Target oligo-nucleotide	Initial fluorescence intensity (A)	Fluorescence intensity after hybridization (B)	(A-B)/A
No. 1	340	350	-0.03
No. 2	332	328	0.01
No. 3	343	336	0.02
No. 4	345	52	0.84

--

Please replace the paragraph at page 147, lines 6-18, as follows:

--In the method of the present invention for analyzing data (for example, the data in columns A and B in Table 7) obtained by the method for analyzing or determining polymorphism and/or mutation of a target nucleic acid (for example, the target oligonucleotide No. 1, 2, 3 or 4), the processing to correct a fluorescence intensity of a reaction system, said fluorescence intensity being obtained when a target nucleic acid is

hybridized with a nucleic acid probe according to the present invention (for example, the above-described nucleic acid probe), by a fluorescence intensity of the same reaction system when the target nucleic acid is not hybridized with the nucleic acid probe means the calculation of (A-B)/A in Table 4.--

Please replace the paragraph at page 149, lines 8-12, as follows:

--Further, arrangement of a minute temperature sensor and a microheater on the opposite side of the slide glass at a position corresponding to each spot of the modified probe makes it possible to provide the DNA chip of the present invention with high performance.--

Please replace the paragraph on page 160, lines 15-17, as follows:

--f) Fluorescence assay (measurement):

Assay (measurement) was performed once after each of denaturation and extension in each cycle.--

Please replace the paragraph at page 176, line 22, to page 177, line 8, as follows:

--Using "BcaBEST™ RNA PCR Kit" (product of Takara Shuzo Co., Ltd., Kyoto, Japan), the extract was subjected with respect to 16s RNA to amplification and reverse transcription reaction (RT-PCR) under known usual conditions in accordance with the protocol of the kit. Upon these amplification and reverse transcription reaction (RT-PCR), the above-described fluorescence quenching probe EU1392R according to the present invention was used as a primer. Subsequently, RNA was cleaved by RNase H (30°C, 20 minutes), and pure cDNA of the 16S rRNA gene was obtained. The concentration of cDNA was determined using "OliGreen^RssDNA Quantitation Kit" (trade name; product of Molecular Probes, Inc., OR, U.S.A.).--

Please replace the paragraph at page 188, line 8, to page 190, line 1, as follows:

--Using the above-described artificial co-cultivation system of microorganisms (the mixed 16S rRNA gene sample) as a target, quantitative PCR was conducted using fluorescence emitting primers dually modified with Texas Red and Dabcyl. Employed as common primers were Eu47F-modi (CITAACACATGCAAGTCG, I: inosine) and Eu1392R (TTGTACACACCGCCCGTCA). Eu47F-modi had a similar base sequence as Eu47F, but the 9th T from the 5' end was modified with Texas Red and the 9th T was modified with Dabcyl. The modifications with Texas Red and Dabcyl were conducted in a similar manner as in Example 7. As a quantitative PCR apparatus, "iCycler" (trade mark, manufactured by Bio-Rad Laboratories, Inc., CA, U.S.A.) was used. The first denature was carried out at 95°C for 60 seconds, and PCR cycles were conducted under the following conditions: denature: 95°C/60 seconds, annealing: 50°C/60 seconds and extension: 72°C/70 seconds. The PCR reaction was terminated in an exponential growth phase such that the initial composition of the genes would not be altered (no PCR bias would be applied). As the concentrations of the primers, Eu47F and Eu1392R were both set at 0.1 µM, respectively, in terms of final concentration. As a DNA polymerase, "TaKaRa Taq™" (trade name, product of Takara Shuzo Co., Ltd., Kyoto, Japan) was used at a concentration of 0.5 unit/20 µL. The concentration of Mg ions was set at 2 mM. dNTP was added to give a final concentration of 2.5 mM, respectively. Using "AntiTaq body" (trade name, product of Clontech Laboratories, Inc., CA, U.S.A.), "hot-start" PCR was conducted following the maker's instruction manual. As a standard sample for the preparation of a working line, an amplified product of the 16S rDNA gene of *E. coli* was used. The preparation of the amplified product of the 16S rDNA gene of *E. coli* was conducted in a similar manner as the above-described artificial co-cultivation system of microorganisms. Subsequent to the preparation of the working line, quantitation of the artificial co-cultivation system of microorganisms was conducted. The

gene concentration of the artificial co-cultivation system of microorganisms was adjusted to give a concentration of 300,000 copies/20 µL in terms of absolute count (20 µL: total amount). Measurement of fluorescence was conducted once after denature and once after extension in each cycle. Similarly to the quenching rate of fluorescence (%), the emitting rate of fluorescence (%) was determined by correcting the intensity of fluorescence after annealing (at the time of hybridization) with the intensity of fluorescence after denaturation (at the time of dissociation).--

Please replace the paragraph at page 190, lines 1-17, as follows:

--A specific calculation formula can be expressed as:

$$F_n: \quad \{(f_{hyb'n}/f_{den'n})/(f_{hyb'n}/f_{den'n})\} \times 100$$

where

F_n : Emitting rate of fluorescence (%) in the nth cycle,

$f_{hyb'n}$: Intensity of fluorescence during extension (hybridization) in the nth cycle,

$f_{den'n}$: Intensity of fluorescence during denaturation (dissociation) in the nth cycle,

$f_{hyb'n}$: Intensity of fluorescence after extension (hybridization) in a cycle (nth cycle) preceding occurrence of an emission of fluorescence from an amplified product, and

$f_{den'n}$: Intensity of fluorescence after denaturation (dissociation) in the cycle (nth cycle) preceding the occurrence of the emission of fluorescence from the amplified product.--

Please replace the paragraph at page 196, lines 7-21, as follows:

--The results of real-time monitoring of the amplified products by the fluorescent emitting probes are shown in FIG. 35. It has been found from this diagram that amplified products can be monitored by using fluorescence emitting probes. Further, a relation

between the number of cycles required to reach a threshold and the count of initially-added DNA is illustrated in FIG. 35. As is readily appreciated from this diagram, it is understood that the number of cycles and the number of copies added initially is in a linear relation. Incidentally, the correlation coefficient at this time was 0.9993 ($R^2 = 0.9993$). Accordingly, it has been found from this diagram that the quantitation of initial copies of a target gene can be accurately achieved from the n^{th} number of a cycle in which the threshold is reached.--

Please replace the paragraph on page 207, line 13 to page 208, line 4, as follows:

--Using as a template the human genome employed in Example 28, PCR was conducted on the DNA chip while using the above-mentioned primers. PCR products were detected by the fixed fluorescence emitting probes or fluorescence emitting probes. The experiment was carried out using the equipment illustrated in FIG. 13. On the DNA chip with the fluorescence emitting probes and fluorescence emitting probes fixed thereon, a solution containing the primers, the template, Taq polymerase, dNTP, $MgCl_2$ and the like was placed. To avoid leakage of the solution, a cover glass was placed over the solution and sealed with a nail varnish. The chip was mounted on a transparent warming plate with a temperature control program stored therein, and a PCR reaction was conducted on the chip. Amplified products were detected in real time by tracking changes in fluorescence from the fixed fluorescence emitting probes and fluorescence quenched probes by the microscope shown in FIG. 13.--

Please replace the paragraph at page 208, lines 5-15, as follows:

--The first denature was conducted at 95°C for 120 seconds, followed by PCR cycles under the following conditions: denaturation: $95^{\circ}\text{C}/60$ sec, annealing: $69^{\circ}\text{C}/60$ sec, and extension: $72^{\circ}/120$ sec. As primer concentrations, the concentrations of the forward primer and reverse primer were both set at $0.5\text{ }\mu\text{M}$ in terms of final concentration. The template

was added at a final concentration of 1.5 ng/ μ L. As the DNA polymerase, "Gene TaqTM" (trade name, product of NIPPON GENE CO., LTD., Tokyo, Japan) was used at a concentration of 0.5 unit/20 μ L. The concentration of Mg ions was set at 2 mM. dNTP was added to give a final concentration of 2.5 mM, respectively.--

Please replace the paragraph at page 208, line 22, to page 210, line 7, as follows:

--The results of the experiment are shown in FIG. 38. It is understood from the diagram that in all the probes, the change in fluorescence increases with the number of cycle. It has, therefore, been demonstrated that gene amplification and real-time detection of the amplified products can be conducted at the same time by the method of the present invention. The results of the preparation of the denaturation curves of the hybridization complex the amplification products and the respective probes are shown in FIG. 39. It is appreciated from the diagram that in all the probes, a significant change in fluorescence was observed as the temperature became lower. This indicates that the fluorescence emitting probes and fluorescence quenching probes hybridized with the corresponding target base sequences. Accordingly, it has been ascertained to be possible to easily monitor the denaturation curves between the probes of the present invention and the target nucleic acids. The denaturation curves between the amplification product and the fluorescence emitting probe and fluorescence quenching probe WIAF-10600 are in substantial conformity with the denaturation curve between the artificially-synthesized, mismatch-free target and the WIAF-10600 probe as obtained in Example 41, thereby indicating that the human genome employed as a template in this Example is 100% complementary with the base sequence of the probe WIAF-10600. The denaturation curves between the amplification product and the fluorescence emitting probe and fluorescence quenching probe WIAF-10578 are in substantial conformity with the denaturation curve between the artificially-synthesized,

mismatch-free target and the WIAF-10578 probe as obtained in Example 41, thereby indicating that the human genome employed as a template in this Example contains a mismatch relative to the base sequence of the probe WIAF-105787. As can be appreciated from the foregoing, it has been found that use of a DNA chip according to the present invention makes it possible to simultaneously conduct an analysis of plural types of SNPs in an amplification product after a genetic amplification is conducted.--

Please replace Table 12 on page 211 as follows:

--Table 12

Used Fluorescence Emitting Probes and Fluorescence Quenching Probes

Probe name	Probe type	Sequence	Position modified by Texas Red as counted from the 5' end (5' end base: 0 th)	Position of Dabcyl as counted from the 5' end
WIAF-10600-No. 1	Fluorescence emitting probe	5'AAGGGGCACGT GCACATGGC3'	6	12
WIAF-10578-No. 2	Fluorescence emitting probe	5'CCTGCAGCAT CATCTGTTAC CTCAC3'	5	11
WIAF-10600-No. 3	Fluorescence quenching probe	5'AAGGGGCACGT GCACATGGC3'	-	-
WIAF-10579-No. 4	Fluorescence quenching probe	5'CCTGCAGCAT CATCTGTTAC CTCAC3'	-	-

IN THE CLAIMS

Please amend the claims as follows:

--44. (Amended) A method for determining a concentration of a target nucleic acid by using PCR, which comprises:

conducting reactions in PCR by using as a primer a nucleic acid probe according to any one of claims 1-17, and

determining an initial concentration of the amplified target nucleic acid from percentage of a change in an intensity of fluorescence occurred as a result of hybridization between said primer or an amplified nucleic acid amplified using said primer and said amplified target nucleic acid.

47. (Amended) A method according to claim 45 or 46 for determining an initial concentration of a nucleic acid amplified in PCR, wherein said PCR is real-time quantitative PCR.

48. (Amended) A method for analyzing data obtained by a determination method according to any one of claims 18-28 or any one of claims 37-47, further comprising correcting an intensity value of fluorescence in a reaction system, said intensity value being available after said target nucleic acid has hybridized to said nucleic acid probe labeled with said fluorescent dye, in accordance with an intensity value of fluorescence in said reaction system available after a probe-nucleic acid hybrid complex so formed has been dissociated.

49. (Amended) A method for analyzing data obtained by a real-time quantitative PCR method according to claim 47, further comprising, as a correction processing step, correcting an intensity value of fluorescence in a reaction system, said intensity being available in each cycle after said amplified nucleic acid has conjugated to said fluorescent dye or after said amplified nucleic acid has hybridized to said nucleic acid probe labeled with

said fluorescent dye, in accordance with an intensity value of fluorescence in said reaction system

available after a nucleic acid-fluorescent dye conjugate or probe-nucleic acid hybrid complex so formed has been dissociated in said cycle.--

SUPPORT FOR AMENDMENTS

The specification has been amended for clarity and to correct typographical errors. Support for these amendments can be found in the substitute specification. No new matter is believed to be introduced by the amendments to the specification.

Claims 44 and 47-49 have been amended for clarity and to correct typographical errors. Support for these amendments can be found in the substitute specification. No new matter is believed to be introduced by the amendments to Claims 44 and 47-49.

REMARKS

Claims 1-54 are active in the present application.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Contents of the paper copy of the substitute Sequence Listing and the computer-readable Sequence Listing are identical. Support for all the sequences listed in the substitute Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute Sequence Listing and the computer-readable Sequence Listing.

Applicants submit herewith a substitute specification and substitute abstract for clarity and for proper numbering, taking into account the newly translated Examples 2 to 8 and Examples 17 to 42. No new matter is believed to be introduced by the substitute specification and the substitute abstract.

The substitute specification has been amended for clarity and to correct typographical errors. No new matter is believed to be introduced by the amendments to the substitute specification.

Claims 44 and 47-49 have been amended for clarity and to correct typographical errors. No new matter is believed to be introduced by the amendments to the Claims.

Applicants submit that this application is now in condition for examination on the merits. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



Norman F. Oblon
Attorney of Record
Registration No. 24,618

Daniel J. Pereira, Ph.D.
Registration No. 45,518



22850

(703) 413-3000

Fax #: (703) 413-2220

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Marked-Up Copy
Serial No:
Amendment Filed on:
NOVEMBER 19, 2001

IN THE SPECIFICATION

Please amend the specification as follows:

Please replace the paragraph at page 7, lines 5-9, as follows:

--[③] Performance of a polymorphous analysis on a target gene LJ after amplifying the target gent by a quantitative gene amplification method makes it possible to easily and quickly determine the pre-amplification amount and polymorphous composition of the target gene with good quantitateness.--

Please replace the paragraph at page 8, lines 3-16, as follows:

--2) A nucleic acid probe for determining a concentration of a target nucleic acid, the probe being labeled with a fluorescent dye, wherein:

the probe is labeled at an end portion thereof with the fluorescent dye, and

the probe has a base sequence designed such that, when the probe hybridizes at the end portion thereof to the target nucleic acid, at least one G (guanine) base exists in a base sequence of the target nucleic acid at a position 1 to 3 bases [apart] from an end base of the target nucleic acid hybridized with the probe;

whereby the fluorescent dye is reduced in fluorescence emission when the probe labeled with the fluorescent dye hybridizes to the target nucleic acid.--

Please replace the paragraph at page 14, lines 11-24, as follows:

--23) A method for determining an initial concentration of a target nucleic acid amplified in PCR, which comprises:

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conducting reactions in PCR by using a nucleic acid probe as described above under any one of 1) to 6);

measuring an intensity of fluorescence in a reaction system in which in a course of a nucleic acid extending reaction, the probe has been degraded out from the probe-target nucleic acid complex by polymerase or in which a nucleic acid denaturing reaction is proceeding or has been completed and also an intensity of fluorescence in the reaction system in which the target nucleic acid or amplified target nucleic acid is hybridized with the nucleic acid probe; and then

calculating percentage of a change in the latter intensity of fluorescence from the former intensity of fluorescence.

Please replace the paragraph at page 52, lines 6-9, as follows:

--The nucleic acid probe according to the present invention [can be readily] can also be readily obtained by ordering its synthesis like the synthesis of the oligonucleotide, provided that only the designing of the probe can be completed.--

Please replace the paragraph at page 52, lines 10-12, as follows:

--A description will next be made about the fluorescence quenching probe according to the second aspect of the present.

This probe is characterized in that it is an oligonucleotide labeled with a single fluorescent dye and, when hybridized with a target nucleic acid, the intensity of its fluorescence decreases. It, therefore, has a property opposite to the fluorescence emitting probe.--

Please replace the paragraph at page 53, line 21, to page 54, line 15, as follows:

--The number of bases in the probe according to the present invention is similar to that in the above-described invention. No particular limitation is imposed on the base

sequence of the probe insofar as it specifically hybridizes to the target nucleic acid. Preferred examples of the base sequence of the probe can include:

(1) a base sequence designed such that at least one G (guanine) base exists in the base sequence of the target nucleic acid at a position 1 to 3 bases [apart] from the end base portion of the target nucleic acid hybridized to the-probe,

(2) a base sequence designed such that plural base pairs of a nucleic acid hybrid complex forms at least one G (guanine) and C (cytosine) pair at an end portion of the probe, and

(3) a base sequence designed such that in the probe modified with the fluorescent label at a portion other than the 5' end phosphate group or the 3' end OH group, base pairs in the fluorescence-labeled portion forms at least one G (guanine) and C (cytosine) pair,

when the nucleic acid probe labeled with the fluorescent dye is hybridized with the target nucleic acid.--

Please amend the paragraph at page 61, line 12, to page 62, line 18, as follows:

--Fundamental operations in the determination method making use of the device according to the present invention are simply to place a solution, which contains a target nucleic acid such as mRNA, cDNA or rRNA, on the solid support on which the nucleic probes are bound and then to induce hybridization. As a result, a change in the intensity of fluorescence takes place corresponding to the concentration of the target nucleic acid, and the target nucleic acid can then be detected and quantitated from the change in the intensity of fluorescence. Further, binding of many nucleic acid probes of different base sequences on a surface of a single support makes it possible to determine concentrations of many target nucleic acids at the same time. As this device can be used for exactly the same application as a DNA chip, that is, for the determination of the concentrations of the target nucleic acids, it is a novel DNA chip. Under reaction conditions optimal for the target nucleic acid, the

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nucleic acids other than the target nucleic acid do not change the intensities of fluorescence emitted from [remain unchanged] the probe. No operation is, therefore, needed for washing off the unreacted nucleic acids. Further, independent temperature control of the individual nucleic acid probes according to the present invention by their corresponding microheaters makes it possible to control the probes under their optimal reaction conditions, respectively. Accurate determination of concentrations is therefore feasible. In addition, a denaturation curve between each nucleic acid probe of this invention and its corresponding target nucleic acid can be analyzed by continuously changing the temperature with the microheater and measuring the intensity of fluorescence during the changing of the temperature. From differences in such denaturation curves, it is possible to determine properties of the hybridized nucleic acid and also to detect SNP.--

Please replace the paragraph at page 66, lines 4-11, as follows:

--In the present invention, the nucleic acid probe is hybridized to the target nucleic acid as described above. The intensity of fluorescence emitted from the fluorescent dye is measured both before and after the hybridization, and a [decrease] change in fluorescence intensity after the hybridization is then calculated. As the [decrease] change is proportional to the concentration of the target nucleic acid, the concentration of the target nucleic acid can be determined.--

Please replace the paragraph at page 74, line 22, to page 75, line 7, as follows:

--The followings can be mentioned as illustrative examples of the fluorescence quenching probe:

(1) A probe labeled, at an portion, preferably, an end thereof, with a fluorescent dye useful in the practice of the present invention. The base sequence of the probe is designed such that, when hybridizes to a target nucleic acid, at least one G (guanine) base exists in the base sequence of the target nucleic acid at a position 1 to 3 bases [apart] from the end base of

the target nucleic acid hybridized on the end portion or end of the probe where the probe is labeled with the fluorescent dye.--

Please replace the paragraph at page 77, line 11, to page 78, line 22, as follows:

--In particular, the above-described probe (7) of the present invention is designed such that it is not used as a primer. PCR is conducted by using a single probe of the present invention as opposed to two (fluorescent-dye-labeled) probes needed in a real-time quantitative PCR method making use of the FRET phenomenon. The probe is added to a PCR reaction system, and PCR is then conducted. During a nucleic acid extending reaction, the probe which has been in a form hybridized with the target nucleic acid or amplified target nucleic acid is degraded by polymerase and is dissociated off from the nucleic acid hybrid complex. The intensity of fluorescence of the reaction system at this time or the reaction system in which a nucleic acid denaturing reaction has completed is measured. Further, the intensity of fluorescence of the reaction system in which the target nucleic acid or amplified target nucleic acid has hybridized with the probe (i.e., the reaction system at the time of an annealing reaction or at the time of the nucleic acid extending reaction until the probe is eliminated from the nucleic acid hybrid complex by polymerase) is measured. By calculating a decrease [of] in the [latter] former fluorescence intensity based on [from] the latter [former] fluorescence intensity, the concentration of the amplified nucleic acid is determined. The intensity of fluorescence is high when the probe has completely dissociated from the target nucleic acid or amplified target nucleic acid by the nucleic acid denaturing reaction or when the probe has been degraded out from the hybrid complex of the probe and the target nucleic acid or amplified nucleic acid at the time of extension of the nucleic acid. However, the intensity of fluorescence of the reaction system in which an annealing reaction has been completed and the probe has fully hybridized to the target nucleic acid or amplified target nucleic acid or of the reaction system until the probe is degraded out of the hybrid complex

of the probe and the target nucleic acid or, amplified target nucleic acid by polymerase at the time of a nucleic acid extending reaction is lower than the former. The decrease in the intensity of fluorescence is proportional to the concentration of the amplified nucleic acid.--

Please replace the paragraph at page 86, line 22, to page 87, line 20, as follows:

--A third feature resides in a data analysis method, which comprises the following processing steps:

1) performing processing in accordance with the following formula (5), (6) or (7) by using data of rates or percentages of changes in fluorescence as calculated in accordance with said formula (3) or (4):

$$\log_b(F_n), \ln(F_n) \quad (5)$$

$$\log_b\{(1-F_n) \times A\}, \ln\{(1-F_n) \times [b] \underline{A}\} \quad (6)$$

$$\log_b\{(F_n-1) \times A\}, \ln\{(F_n-1) \times A\} \quad (7)$$

where

A, b: desired numerical values, preferably integers, more preferably natural numbers and, when A=100, b=10, $\{(F_n-1) \times A\}$ is expressed in terms of percentage (%), and

F_n : rate or percentage of a change in fluorescence in an n^{th} cycle as calculated in accordance with the formula (3) or formula (4),

2) determining a cycle in which said processed value of said processing step 1) has reached a constant value,

3) calculating a relational expression between cycle of a nucleic acid sample of a known concentration and the number of copies of said target nucleic acid at the time of initiation of a reaction, and

4) determining the number of copies of said target nucleic acid in an unknown sample upon initiation of PCR.--

Please replace the paragraph at page 92, lines 15-24, as follows:

--The feature of the polymorphous analysis method according to the present invention resides in the use of the nucleic acid probe of this invention in a conventional polymorphous analysis method to [determination] determine a nucleic acid. The term "polymorphous" or "polymorphism" as used herein means biological polymorphous or polymorphism. In the present invention, it means especially the polymorphism of a gene (RNA, DNA, gene) on which the polymorphism is brought about. It has the same meaning as commonly employed these days in molecular biology.--

Please replace the paragraph at page 94, line 25, to page 95, line 9, as follows:

--For example, as has been described in detail in connection with the second aspect of the invention, a fluorescence quenching probe is labeled at an end thereof with a fluorescent dye, and its base sequence is designed such that, when the probe hybridizes at the end portion thereof to a target gene, at least one G (guanine) base exists in a base sequence of the target gene at a position 1 to 3 bases [apart] from the portion of an end base pair of the target gene hybridized with the probe, whereby the fluorescent dye is reduced in fluorescence emission when the probe hybridizes to the target gene.--

Please replace the paragraph at page 99, lines 7-12, as follows:

--It is preferred to prepare a working line for the target gene by using [a] an authentic target gene before the amplifying reaction of the target gene. A description will now be made about an illustrative case in which the above-described fluorescence quenching probe was used as a primer and the real-time monitoring quantitative PCR method was conducted.--

Please replace the paragraph at page 101, line 22, to page 102, line 1, as follows:

--(2) Gene fragments digested as described above can preferably be thermally [modified] denatured into single-stranded forms. This [modification] denaturation treatment can be conducted under usual conditions known to the public. For example, they are treated at 97°C for 5 minutes and then chilled in ice.--

Please replace the paragraph at page 107, line 20, to page 108, line 3, as follows:

--The invention [nuclear] nucleic acid probe synthesized as described above is free of any base sequence having complementation at at least two positions between the base chains at positions where the probe was labeled with Texas Red as a fluorescent dye and Dabcyl as a quencher substance, respectively. The invention [nuclear] nucleic acid probe, therefore, does not form any double-stranded chain in its own chain. In other words, the invention [nuclear] nucleic acid probe does not form any stem-loop structure.--

Please replace the paragraph at page 111, line 25, to page 113, line 1, as follows:

--The results are diagrammatically shown in FIG. 4. As is appreciated readily from the diagram, it was observed that in most of the fluorescence emitting probes of the present invention dually modified by Dabcyl and Texas Red, hybridization with the target deoxyribooligonucleotide leads to an increase in the emission of fluorescence compared with the emission of fluorescence before the hybridization. Further, maximum emission of fluorescence was observed when the inter-base distance from the base having the phosphate group labeled with Texas Red to the Dabcyl-labeled base (when counted by assuming that the base number of the base labeled with Text Red was the 0th base) was 6 bases long. The emission of fluorescence at that time was about 11 times higher compared with that before hybridization. When the inter-base distance was 16 bases long, large emission of fluorescence was also observed. The emission of fluorescence at that time was about 11 times as in the case of the 6 bases. As a DNA helix makes a turn with 10 bases, the 6th and 16th bases of a double-stranded DNA structure as observed from the 5' end base are located substantially on the opposite side of the helix. It is therefore considered that, when the 6th and 16th bases were labeled with the quencher substance, quenching of fluorescence took place based on transfer of electrons between Dabcyl and Texas Red when the deoxyribooligonucleotide is in the single-stranded form, but as a result of physical separation

of Dabcyl and Texas Red from each other by the hybridization, the quenching of fluorescence based on the transfer of electrons was canceled and Texas Red emitted fluorescence.--

Please replace the paragraph at page 113, line 13, to page 114, line 2, as follows:

--An absorption of Dabcyl as a quencher appears at 400 to 500 nm. Many of probes with large emission of fluorescence, however, emitted fluorescence at wavelengths substantially shifted from the absorption of Dabcyl, that is, at wavelengths longer than 550 nm. In the case of a fluorescent dye which emits fluorescence at wavelengths longer than 550 nm, the mechanism of fluorescence quenching by Dabcyl is considered to be attributable primarily to transfer of photoexcited electrons rather than FRET. Since Dabcyl and the fluorescent dye are physically separated from each other as a result of a change in the stereostructure of the [probe] double-stranded nucleic acids by hybridization, the fluorescence quenching phenomenon by the transfer of photoexcited electrons is cancelled. In the case of a fluorescent dye such as FITC which emits fluorescence at wavelengths close to the absorption of Dabcyl, it is considered that, even when Dabcyl and a fluorescent dye are physically separated as a result of a change in the stereostructure of the double-stranded nucleic acid and the fluorescence quenching phenomenon based on transfer of photoexcited electrons is hence cancelled, no substantial emission of fluorescence takes place from quenching of fluorescence by FRET because the quenching of fluorescence by FRET prevails. Accordingly, a dye capable of satisfying the following three conditions is desired as a fluorescent dye for use in a fluorescence emitting probe according to the present invention: (1) the fluorescence quenching phenomenon based on transfer of photoexcited electrons occurs between the fluorescent dye and Dabcyl; (2) Fluorescence of wavelengths substantially shifted from the absorption of Dabcyl is emitted; and (3) a strong interaction exists between the fluorescent dye and Dabcyl to reduce the intensity of fluorescence before

hybridization (in other words, to further facilitate the occurrence of the fluorescence quenching phenomenon by transfer of photoexcited electrons).--

Please replace the paragraph at page 133, line 20, to page 134, line 2, as follows:

--Using a DNA synthesizer, an oligoribonucleotide having a base sequence of (5')TCCTTTGAGT TCCCGGCCGG(3') A was synthesized as in the above to provide it as a forward-type [hepter] helper probe. On the other hand, an oligoriboxynucleotide having a base sequence of (5')CCCTGGTCGT AAGGGCCATG ATGACTTGAC GT (3') was synthesized by using a DNA synthesizer, in a similar manner as described above to provide it as a backward-type, in other words, reverse-type helper probe.--

Please replace the paragraph at page 145, line 15, to page 146, line 15, as follows:

--Four oligonucleotides with the below-described base sequences were synthesized using the same DNA synthesizer as that employed in Example 12. Further, an invention nucleic acid probe having the below-described base sequence was also synthesized in a similar manner as in Example 12. The target oligonucleotides were separately hybridized with the probe in solutions. An investigation was then made as to whether or not a single base substitution can be determined from a change in fluorescence intensity. The base sequence of the nucleic acid probe according to the present invention is designed such that, if G exists at the 3' end of any one of the target oligonucleotides, it matches 100% with the base sequence of the particular oligonucleotide. The hybridization temperature was set at 40°C at which all base pairs between the probe and the target oligonucleotide can hybridize 100%. The concentrations of the probe and target oligonucleotides, the concentration of a buffer solution, a fluorimeter, fluorescence measuring conditions, experimental procedures, and the like were set or chosen as in Example 12.--

Please replace Table 7 on page 147 as follows:

--Table 7

Target oligo-nucleotide	Initial fluorescence intensity (A)	Fluorescence intensity after hybridization (B)	$(A-B)/[B]\Delta$
No. 1	340	350	-0.03
No. 2	332	328	0.01
No. 3	343	336	0.02
No. 4	345	52	0.84

--

Please replace the paragraph at page 147, lines 6-18, as follows:

--In the method of the present invention for analyzing data (for example, the data in columns A and B in Table 7) obtained by the method for analyzing or determining polymorphism and/or mutation of a target nucleic acid (for example, the target oligonucleotide No. 1, 2, 3 or 4), the processing to correct a fluorescence intensity of a reaction system, said fluorescence intensity being obtained when a target nucleic acid is hybridized with a nucleic acid probe according to the present invention (for example, the above-described nucleic acid probe), by a fluorescence intensity of the same reaction system when the target nucleic acid is not hybridized with the nucleic acid probe means the calculation of $(A-B)/[B]\Delta$ in Table 4.--

Please replace the paragraph at page 149, lines 8-12, as follows:

--Further, arrangement of a minute temperature sensor and a microheater on the [lower] opposite side of the slide glass at a position corresponding to each spot of the modified probe makes it possible to provide the DNA chip of the present invention with high performance.--

Please replace the paragraph on page 160, lines 15-17, as follows:

--f) Fluorescence assay (measurement):

Assay (measurement) was performed once after each of denaturation and [annealing] extension in each cycle.--

Please replace the paragraph at page 176, line 22, to page 177, line 8, as follows:

--Using "BcaBEST™ RNA PCR Kit" (product of Takara Shuzo Co., Ltd., Kyoto, Japan), the extract was subjected with respect to 16S RNA to amplification and reverse transcription reaction (RT-PCR) under known usual conditions in accordance with the protocol of the kit. Upon these amplification and reverse transcription reaction (RT-PCR), the above-described fluorescence quenching probe EU1392R according to the present invention was used as a primer. Subsequently, RNA was cleaved by [Rnase] RNase H (30°C, 20 minutes), and pure cDNA of the 16S rRNA gene was obtained. The concentration of cDNA was determined using "OliGreen^RssDNA Quantitation Kit" (trade name; product of Molecular Probes, Inc., OR, U.S.A.).--

Please replace the paragraph at page 188, line 8, to page 190, line 1, as follows:

--Using the above-described artificial co-cultivation system of microorganisms (the mixed 16S rRNA gene sample) as a target, quantitative PCR was conducted using fluorescence emitting primers dually modified with Texas Red and Dabcyl. Employed as common primers were Eu47F-modi (CITAACACATGCAAGTCG, I: inosine) and Eu1392R (TTGTACACACCGCCCGTCA). Eu47F-modi had a similar base sequence as Eu47F, but the 9th T from the 5' end was modified with Texas Red and the 9th T was modified with Dabcyl. The modifications with Texas Red and Dabcyl were conducted in a similar manner as in Example 7. As a quantitative PCR apparatus, "iCycler" (trade mark, manufactured by Bio-Rad Laboratories, Inc., CA, U.S.A.) was used. The first denature was carried out at 95°C for 60 seconds, and PCR cycles were conducted under the following conditions: denature: 95°C/60 seconds, annealing: 50°C/60 seconds and extension: 72°C/70 seconds. The PCR reaction was terminated in an exponential growth phase such that the initial composition of the genes would not be altered (no PCR bias would be applied). As the concentrations of the primers, Eu47F and Eu1932R were both set at 0.1 μM, respectively, in

terms of final concentration. As a DNA polymerase, "TaKaRa [Taq™] Taq™" (trade name, product of Takara Shuzo Co., Ltd., Kyoto, Japan) was used at a concentration of 0.5 unit/20 µL. The concentration of Mg ions was set at 2 mM. dNTP was added to give a final concentration of 2.5 mM, respectively. Using "AntiTaq body" (trade name, product of Clontech Laboratories, Inc., CA, U.S.A.), "hot-start" PCR was conducted following the maker's instruction manual. As a standard sample for the preparation of a working line, an amplified product of the 16S rDNA gene of *E. coli* was used. The preparation of the amplified product of the 16S rDNA gene of *E. coli* was conducted in a similar manner as the above-described artificial co-cultivation system of microorganisms. Subsequent to the preparation of the working line, quantitation of the artificial co-cultivation system of microorganisms was conducted. The gene concentration of the artificial co-cultivation system of microorganisms was adjusted to give a concentration of 300,000 copies/20 µL in terms of absolute count (20 µL: total amount). Measurement of fluorescence was conducted once after denature and once after [annealing] extension in each cycle. Similarly to the quenching rate of fluorescence (%), the emitting rate of fluorescence (%) was determined by correcting the intensity of fluorescence after annealing (at the time of hybridization) with the intensity of fluorescence after denaturation (at the time of dissociation).--

Please replace the paragraph at page 190, lines 1-17, as follows:

--A specific calculation formula can be expressed as:

$$F_n: \quad \{(f_{hyb'n}/f_{den'n})/(f_{hyb'n}/f_{den'n})\} \times 100$$

where

F_n : Emitting rate of fluorescence (%) in the nth cycle,

$f_{hyb/n}$: Intensity of fluorescence during [annealing] extension (hybridization) in the n^{th} cycle,

$f_{den'n}$: Intensity of fluorescence during denaturation (dissociation) in the n^{th} cycle,

f_{hyb^n} : Intensity of fluorescence after [annealing] extension (hybridization) in a cycle (n^{th} cycle) preceding occurrence of an emission of fluorescence from an amplified product, and

f_{den^n} : Intensity of fluorescence after denaturation (dissociation) in the cycle (n^{th} cycle) preceding the occurrence of the emission of fluorescence from the amplified product.--

Please replace the paragraph at page 196, lines 7-21, as follows:

--The results of real-time monitoring of the amplified products by the fluorescent emitting probes are shown in FIG. 35. It has been found from this diagram that amplified products can be monitored by using fluorescence emitting probes. Further, a relation between the number of cycles required to reach a threshold [$\log F_n$ (emitting rate of fluorescence, %) = 1.8] and the count of initially-added DNA is illustrated in FIG. 35. As is readily appreciated from this diagram, it is understood that the number of cycles and the number of copies added initially is in a linear relation. Incidentally, the correlation coefficient at this time was 0.9993 ($[R^2] R^2 = 0.9993$). Accordingly, it has been found from this diagram that the quantitation of initial copies of a target gene can be accurately achieved from the n^{th} number of a cycle in which the threshold is reached.--

Please replace the paragraph on page 207, line 13 to page 208, line 4, as follows:

--Using as a template the human genome employed in Example 28, PCR was conducted on the DNA chip while using the above-mentioned primers. PCR [modification] products were detected by the fixed fluorescence emitting probes or fluorescence emitting probes. The experiment was carried out using the equipment illustrated in FIG. 13. On the DNA chip with the fluorescence emitting probes and fluorescence emitting probes fixed thereon, a solution containing the primers, the template, Taq polymerase, dNTP, MgCl_2 and the like was placed. To avoid leakage of the solution, a cover glass was placed over

the solution and sealed with a nail varnish. The chip was mounted on a transparent warming plate with a temperature control program stored therein, and a PCR reaction was conducted on the chip. Amplified products were detected in real time by tracking changes in fluorescence from the fixed fluorescence emitting probes and fluorescence quenched probes by the microscope shown in FIG. 13.--

Please replace the paragraph at page 208, lines 5-15, as follows:

--The first denature was conducted at 95°C for 120 seconds, followed by PCR cycles under the following conditions: denaturation: 95°C/60 sec, annealing: 69°C/60 sec, and extension: 72°/120 sec. As primer concentrations, the concentrations of the forward primer and reverse primer were both set at 0.5 μ M in terms of final concentration. The template was added at a final concentration of 1.5 ng/ μ L. As the DNA polymerase, "Gene [TaqTM] TaqTM" (trade name, product of NIPPON GENE CO., LTD., Tokyo, Japan) was used at a concentration of 0.5 unit/20 μ L. The concentration of Mg ions was set at 2 mM. dNTP was added to give a final concentration of 2.5 mM, respectively.--

Please replace the paragraph at page 208, line 22, to page 210, line 7, as follows:

--The results of the experiment are shown in FIG. 38. It is understood from the diagram that in all the probes, the change in fluorescence increases with the number of cycle. It has, therefore, been demonstrated that gene amplification and real-time detection of the amplified products can be conducted at the same time by the method of the present invention. The results of the preparation of the denaturation curves [between] of the hybridization complex the amplification products and the respective probes are shown in FIG. 39. It is appreciated from the diagram that in all the probes, a significant change in fluorescence was observed as the temperature became lower. This indicates that the fluorescence emitting probes and fluorescence quenching probes hybridized with the corresponding target base

sequences. Accordingly, it has been ascertained to be possible to easily monitor the denaturation curves between the probes of the present invention and the target nucleic acids. The denaturation curves between the amplification product and the fluorescence emitting probe and fluorescence quenching probe WIAF-10600 are in substantial conformity with the denaturation curve between the artificially-synthesized, mismatch-free target and the WIAF-10600 probe as obtained in Example 41, thereby indicating that the human genome employed as a template in this Example is 100% complementary with the base sequence of the probe WIAF-10600. The denaturation curves between the amplification product and the fluorescence emitting probe and fluorescence quenching probe WIAF-10578 are in substantial conformity with the denaturation curve between the artificially-synthesized, mismatch-free target and the WIAF-10578 probe as obtained in Example 41, thereby indicating that the human genome employed as a template in this Example contains a mismatch relative to the base sequence of the probe WIAF-10578. As can be appreciated from the foregoing, it has been found that use of a DNA chip according to the present invention makes it possible to simultaneously conduct an analysis of plural types of SNPs in an amplification product after a genetic amplification is conducted.--

Please replace Table 12 on page 211 as follows:

--Table 12

Used Fluorescence Emitting Probes and Fluorescence Quenching Probes

Probe name	Probe type	Sequence	Position modified by Texas Red as counted from the 5' end (5' end base: 0 th)	Position of Dabcyl as counted from the 5' end
WIAF-10600-No. 1	Fluorescence emitting probe	5'AAGGGCACGT GCACATGGC3'	6	12
WIAF-10578-No. 2	Fluorescence emitting probe	5'CCTGCAGCAT CATCTGTTAC CTCAC3'	5	11
WIAF-10600-No. 3	Fluorescence [emitting] quenching probe	5'AAGGGCACGT GCACATGGC3'	[9] =	[15] =
WIAF-10579-No. 4	Fluorescence [emitting] quenching probe	5'CCTGCAGCAT CATCTGTTAC CTCAC3'	[5] =	[11] =

IN THE CLAIMS

Please amend the claims as follows:

--44. (Amended) A method for determining a concentration of a target nucleic acid by using PCR, which comprises:

conducting reactions in PCR by using as a primer a nucleic acid probe according to any one of claims 1-17, and

determining an initial concentration of the amplified target nucleic acid from percentage of a change in an intensity of fluorescence occurred as a result of hybridization between said primer or an amplified nucleic acid amplified [from] using said primer and said amplified target nucleic acid.

47. (Amended) A method according to claim 45 or 46 for determining [a] an initial concentration of a nucleic acid amplified in PCR, wherein said PCR is real-time quantitative PCR.

48. (Amended) A method for analyzing data obtained by a determination method according to any one of claims 18-28 or any one of claims 37-47, further comprising correcting an intensity value of fluorescence in a reaction system, said intensity value being available after said target nucleic acid has hybridized to said nucleic acid probe labeled with said fluorescent dye, in accordance with an intensity value of fluorescence in said reaction system available after a probe-nucleic acid hybrid complex so formed has been [denatured] dissociated.

49. (Amended) A method for analyzing data obtained by a real-time quantitative PCR method according to claim 47, further comprising, as a correction processing step, correcting an intensity value of fluorescence in a reaction system, said intensity being available in each cycle after said amplified nucleic acid has conjugated to said fluorescent dye or after said amplified nucleic acid has hybridized to said nucleic acid probe labeled with

said fluorescent dye, in accordance with an intensity value of fluorescence in said reaction system

available after a nucleic acid-fluorescent dye conjugate or probe-nucleic acid hybrid complex so formed has been [denatured] dissociated in said cycle.--

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